Plant Mol Biol (2008) 67:629–642
DOI 10.1007/s11103-008-9343-3

Barley (*Hordeum vulgare* L.) inositol monophosphatase: gene structure and enzyme characteristics

Jianming Fu · Kevin Peterson · Mary Guttieri · Edward Souza · Victor Raboy

Received: 7 August 2007 / Accepted: 4 May 2008 / Published online: 21 May 2008

Abstract The cellular *myo*-inositol (Ins) pool is important to many metabolic and signaling pathways in plants. Ins monophosphatase (IMPase; EC 3.1.3.25) activity is essential for the de novo synthesis of *myo*-Inositol (Ins), and for recycling of Ins in Ins(1,4,5)P_3. However, proteins encoded by at least one family of *IMP* genes also have L-galactose-1-P phosphatase activity important to ascorbic acid synthesis, indicating a bifunctionality that links these two branches of carbon metabolism. As part of research into the regulation of Ins synthesis and supply during seed development, the barley *IMP*-1 gene and gene products were studied. The 1.4 kb barley *IMP*-1 promoter contains one low temperature response element (RE), two heat shock REs, one gibberellin and two auxin REs, and five sugar REs. Barley *IMP*-1 is expressed in all tissues assayed, and expression levels were not greatly altered by abiotic stress treatments. Reduced use of Ins for Ins P_6 synthesis in developing seed of barley *low phytic acid* (*lpa*) mutants results in Ins accumulation, and *IMP*-1 expression is reduced in proportion to the increase in Ins level. The barley recombinant enzyme had a lower K_m, indicating higher affinity, for D/L-Ins(3)P_1 (K_m = 9.7 μM) as compared with reported K_m (Ins P_1) values for other eukaryotic IMPases (43–330 μM) or with a reported K_m (L-Gal-1P) of 150 μM for a kiwifruit (*Actinidia deliciosa*) enzyme. These and other data indicate that the barley *IMP*-1 gene is regulated at least in part in response to Ins metabolic needs, and that the enzyme it encodes displays catalytic properties well suited for a role in Ins synthesis, in addition to other roles as an L-gal-1-P phosphatase important to ascorbate synthesis, or as an IMPase important to Ins(1,4,5)P_3 signal recycling.

Keywords Inositol · Phytic acid · Seed development

Abbreviations

CTAB	Hexadecyltrimethylammonium bromide
HEPES	*N*-2-Hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid
Ins	*myo*-Inositol
lpa	*Low phytic acid*
Ins P_6	*myo*-Inositol-1,2,3,4,5,6-hexakisphosphate
IPTG	Isopropyl β-D-thiogalactoside
MIPS	*myo*-Inositol-3-phosphate synthase
IMPase	*myo*-Inositol monophosphatase

The barley *IMP*-1 cDNA and protein sequence accession number is AY460570. Accession numbers for the barley genomic sequence including promoter and coding region are DQ145527 and DQ145528. These sequences will be available in GenBank upon the publication of this paper.

J. Fu · M. Guttieri · E. Souza
Research and Extension Center, University of Idaho, 1699 S 2700 W, Aberdeen, ID 83210, USA

Present Address:
J. Fu
Department of Agronomy, Kansas State University, Manhattan, KS 66506, USA

K. Peterson · V. Raboy (✉)
USDA-ARS, 1691 South 2700 West, Aberdeen, ID 83210, USA
e-mail: victor.raboy@ars.usda.gov

Present Address:
K. Peterson
Mayo Clinic, 1216 Second Street SW, Rochester, MN 55902, USA

Present Address:
M. Guttieri · E. Souza
USDA-ARS, 1680 Madison Ave., Wooster, OH 44691, USA

Introduction

myo-Inositol (Ins), the six-carbon cyclitol derivative of glucose, represents a cellular pool essential to Ins phosphate and phosphatidylinositol phosphate pathways important in housekeeping and signaling in eukaryotic cells. The de novo biosynthesis of Ins is via a two-step pathway first described by Chen and Charalampous (1966). It is catalyzed by D- *myo*-inositol-3-P_1 synthase (MIPS, EC 5.5.1.4), which converts glucose-6 -phosphate to D-Ins(3)P_1,[1] which is then converted to Ins by Ins monophosphatase (IMPase, EC 3.1.3.25).

The cellular Ins pool is important in several functions unique to plant biology (Loewus and Murthy 2000). Seeds store 70 ± 10% of their total P as Ins P_6 ("phytic acid"), and thus seed Ins P_6 represents a major bottleneck in P flux through the agricultural ecosystem (Raboy 2007). Several lines of evidence indicate that de novo synthesis of Ins, catalyzed by MIPS and IMPase, and its conversion either to PtdIns or back to Ins(3)P_1, contributes to Ins P_6 accumulation during seed development (Hitz et al. 2002; Shi et al. 2005; Yoshida et al. 1999). Additional Ins-requiring functions unique to plant cells include: oxidation to D-glucuronic acid, catalyzed by Ins oxygenase (MIOX), important to the biogenesis of uronosyl and pentosyl units of cell wall polysaccharides (e.g. pectin and hemicelluloses); conjugation to form Ins-auxin (IAA), an inactive form of IAA that may facilitate the auxin long-distance transport from shoots to roots within the plant (Cohen and Bandurski 1982); methylation to D-ononitols and D-pinitols, involved in osmoprotection and oxygen radical scavenging (Ishitani et al. 1996; Smirnoff and Cumbes 1989).

Ins and molecules derived from it are involved in stress responses in a number of species. In response to drought, Ins increased significantly in both leaves and stems in *Vigna umbellata* (Wanek and Richter 1997). A cytosolic MIPS is up-regulated by the salinity conditions in the common ice plant (Ishitani et al. 1996; Nelson et al. 1998). Recently a novel salt-tolerant MIPS, encoded by the *PINO1* gene, was isolated from the halophytic wild rice, *Porteresia coarctata* (Roxb.) Tateoka (Majee et al. 2004). Ins-*o*-methyltransferase (IMT1) mRNA is also up-regulated under the salinity condition in the common ice plant (Ishitani et al. 1996; Nelson et al. 1998). The proposed pathway in response to abiotic stress is that MIPS and IMPase convert glucose-6-P to Ins, IMT1 converts Ins to D-ononitol which is further converted to D-pinitol by D-inositol epimerase. D-ononitol and D-inositol serve as osmoprotectants and oxygen radical scavengers. IMPase may also function in "signal termination"; recycling Ins ultimately derived from Ins(1,4,5)P_3 produced during signal transduction.

As an outcome of interest in mammalian brain IMPase as the site of action for Li^+ bipolar disorder therapy (Berridge et al. 1989), the first IMPase-encoding sequence was isolated from bovine brain tissue (Diehl et al. 1990). A subsequent study of mammalian (bovine, human, rat) brain IMPase found that it also has galactose 1-P phosphatase activity, with similar velocity against Gal-1-P as against Ins(1)P_1 (Parthasarathy et al. 1997). During more recent studies of the ascorbic acid biosynthetic pathway in plants, an L-Gal-1-P phosphatase was purified from kiwifruit (*Actinidia deliciosa*), and found to be encoded by a plant homologue of the mammalian *IMP* (Laing et al. 2004). It was then shown that an *Arabidopsis* low-ascorbate locus, *VTC4*, encodes an L-Gal-1Pase/IMPase (Conklin et al. 2006). Thus, in both mammalian and plant tissues, the protein encoded by *IMP* genes might be bifunctional, linking Ins and galactose metabolism.

Phylogenetic analyses (Torabinejad and Gillaspy 2006) indicate that plant genomes typically contain one to several copies of IMPase-encoding *IMP* genes ancestrally related to, and encoding proteins with similar properties to the mammalian *IMP*. In addition, plant genomes also typically contain one or more families of IMPase-encoding sequences more related to prokaryotic *IMPases* than to the mammalian *IMP* which have been referred to as "*IMP*-like" genes (Torabinejad and Gillaspy 2006). Studies and comparisons of plant IMPase biochemical and catalytic properties are relatively limited in number (Gillaspy et al. 1995; Islas-Flores and Villaneuva 2007). As part of the study of Ins metabolism in grain crop species, MIPS genes from barley (*Hordeum vulgare* L.) and maize (*Zea mays* L.) were cloned and chromosomally mapped (Larson and Raboy 1999). Here are reported the initial characterization of the genomic sequence and expression of the barley genome's single *IMP*-1 gene, and the catalytic properties of the enzyme it encodes.

Materials and methods

Plant materials

All studies were conducted with the barley cv. Harrington and four near-isogenic lines (isolines) in the Harrington genetic background, each homozygous for one of four barley *low phytic acid* (*lpa*) single-gene recessive alleles; *lpa*1-1, *lpa*2-1, *lpa*3-1 and M955. These recessive alleles were induced in the Harrington genetic background following chemical mutagenesis, as described (Dorsch et al. 2003). Each allele was then backcrossed to non-mutagenized

[1] With the exception of the use of "D/L" mixtures used in enzyme assays, all Ins phosphates will be referred to here using the "D"-numbering convention; D-Ins(3)P_1 is identical to L-Ins(1)P_1.

Harrington, and following the BC_4 (*lpa*1-1), BC_3 (*lpa*2-1 and *lpa*3-1) and BC_2 (M955), isolines homozygous for a given allele were identified. Plants were gown in a routinely maintained greenhouse.

Total RNA extraction, mRNA purification and cDNA library construction

Plant materials were frozen quickly in liquid N_2 after collection and stored at −80°C until use. Total RNA for quantitative real-time (RT)-PCR was isolated from barley leaves, roots and early-stage developing seeds (estimated to be 10 days after pollination, or 10 DAP, based on the date of the barley head emergence), respectively. RNA for quantitative real-time RT-PCR was treated with DNase (Ambion Inc., Austin, TX, USA). Total RNA for expression library construction was isolated from pooled wild type seeds collected at the early, middle and later stages of development, ranging from 7 DAP to 35 DAP, using the guanidine-based method (Chirgwin et al. 1979). The RNA integrity was verified by agarose gel electrophoresis. The mRNA was enriched using the Poly(A)Purist kit (Ambion Inc., Austin, TX, USA). Double-stranded cDNA was synthesized, and an expression library was constructed using SMART cDNA Library Construction System (BD Biosciences Clontech, Palo Alto, CA, USA) according to the manufacturer's recommendation. Briefly, the first and the second strand cDNA syntheses were directed by Sfi IB-Oligo-d(T) and Sfi IA-oligo primers, respectively. cDNA less than 500 bp in length was removed with CHROMA SPIN-400 column. The double stranded cDNA was digested with restriction enzyme Sfi I to produce two asymmetric ends that were directionally ligated with the Sfi-digested LamdaTripleEx2 vector. The recombinant lambda phage DNA was packaged using Gigapack III Gold Packaging Extract (Strategene, La Jolla, CA, USA). Approximately 10^6 plaque forming unit (pfu) of the primary phage library was converted to plasmid library hosted in *E. coli* strain BM25.8.

Expression library screening, *IMP* cDNA isolation and sequencing

Forward primers located in the vector and reverse primers located in the putative barley *IMP* 3′-UTR were used to amplify the *IMP* 5′-UTR, coding region and partial 3′-UTR from the cDNA library. The completed 3′-UTR and the polyA region was amplified using a barley IMP-specific primer in the coding region, and a reverse primer in the vector (Table 1). PCR reactions contained 0.4 μM of each primer, 0.4 mM dNTPs, 0.1 μg of library plasmids, and 1 unit of pfx DNA polymerase (Invitrogen Corp., California, USA). The PCR program was conducted with 35 cycles of denaturing at 95°C for 30 s, annealing at 57°C for 47 s, and extension at 68°C for 2 min. The PCR products were gel-purified using Qiagen gel purification kit (Qiagen, Germany), cloned in the pCR4-TOPO cloning vector (Invitrogen Corp.), and sequenced using the BigDye method by Sequetech Corp. (California, USA).

The complete *IMP* 5′-sequence was determined by rapid amplification of cDNA ends (RACE) using the RLM-RACE kit (Ambion Inc., Austin, Texas, USA) with

Table 1 Primers and adapters for amplification of barley *IMP*-1 cDNA and genomic sequences

Target sequence description; size	Priming direction	Sequence (5′ → 3′)	Oligo position
CDNA			
Partial 5′-UTR → coding region → 3′-UTR; 1,056 bp	Forward	GGAAGCCATGGCGGAGGAGCAGTTCCTG	−7
	Reverse	CGACTCACTATAGGGCGAATTGGCCAAGTG	In vector
5′-RACE RNA adapter	N/A	GCUGAUGGCGAUGAAUGAACACUGCGUUUGCUGGCUUUGAUGAAA	N/A
5′-RACE reverse transcription	Reverse	CGTCTCCTCGCCGATGAACTTGTGGTCC	207
5′-RACE PCR	Forward	CGCGGATCCGAACACTGCGTTTGCTGGCTTTGATG	N/A
	Reverse	CTTGTGGTCCGGGTAGAGCATCCGGAGGTG	159
Genomic			
5′-Region; 524 bp	Forward	GCAAGCCATGGCGGAGGAGCAGTTCCTG	−7
	Reverse	GTGGTGCCATCGAGGGGGTCGACTATCCAG	288
Middle region; 429 bp	Forward	GTGTGCGTCTCGATTGGCCTCACCATTGG	314
	Reverse	CCTACCTCTGTCACCATAAGAGCCTTCACC	490
3′-Region; 807 bp	Forward	GAGACAAGTCCACTTTGGATGATACAACC	450
	Reverse	AGGGGAGAATTGTAGCTTACGAGCCATTTCA	883

Primers for genomic sequences were designed based on putative exons in the cDNA sequence. Oligonucleotide position is relative to translation initiation codon of the *IMP*-1 cDNA. N/A, not apply

modification. A RACE RNA adapter (5′-RACE RNA Adapter, Table 1) was ligated to the 5′-end of barley mRNA. One *IMP*-specific primer (5′-RACE Reverse Transcription primer, Table 1) was used to prime transcription with SuperScript III Reverse Transcriptase (Invitrogen) at 55°C. The 5′-RACE PCR Forward primer (Table 1) and an *IMP*-specific down-primer (5′-RACE PCR Reverse Adapter, Table 1) were used to amplify the complete *IMP* 5′-UTR in a PCR reaction. The PCR products were gel-purified and sequenced.

IMP cDNA sub-cloning, protein induction, purification, and catalytic properties

The *IMP* coding region was PCR-amplified using pfx DNA polymerase (Invitrogen) and the *IMP* cDNA in pCR4-TOPO vector as a template, and ligated to pET-28b(+) vector (Novagen, Madison, Wisconsin, USA) at the *Nco*I site, such that it has a C-terminus 6× His tag that is co-expressed. The plasmid was transformed into *E. coli* strain BL21(DE3). The junction regions and reading frames were confirmed by sequencing. The *E. coli* cells were cultured until an OD_{600} reached 1.0, and then IPTG was added to a final concentration of 0.4 mM. The cells were allowed to grow overnight and were harvested by centrifugation at 3,000*g*. The cell pellet was resuspended and homogenized in 50 mM Tris pH 7.5 containing 500 mM NaCl while on ice. The crude lysate from the IPTG-induced cells was loaded onto a Ni-NTA column pre-equilibrated with an equilibration buffer containing 50 mM Tris pH 7.5 containing 500 mM NaCl. The column was washed with five column-volumes of equilibration buffer containing 20 mM imidazole, and the recombinant barley IMPase was eluted using 50 mM Tris pH 7.5 containing 500 mM NaCl and 250 mM imidazole. The eluate was concentrated using a centricon-10 centrifugation filter, and the concentrate was dialyzed against three changes of 50 mM HEPES pH 7.5 with 10% glycerol.

IMPase activity assays were conducted in 300 µl reaction mixtures containing 50 mM HEPES pH 7.5, 500 µM D/L-Ins(3)P_1 (Sigma), 25 nCi ^{14}C-labeled D-Ins(3)P_1 (Amersham Life Sciences, Piscataway NJ), 3 mM $MgCl_2$ and 1.0 µg purified barley IMPase. Protein concentrations were determined by the method of Bradford (1976) with bovine serum albumin as standard. The reaction was incubated at 37°C for 10 min, and stopped with the addition of 50 µl of 10% TCA. Reaction substrate and products were fractionated and detected using an anion-exchange HPLC equipped with a flow-through scintillation counter, as follows. Aliquots (100 µl) of assay mixture were loaded onto a Waters (Waters Inc., Millford, MA, USA) strong anion exchange (SAX) column (4.6 × 150 mm) and eluted with a 20 min gradient of 1.0–50 mM K_2HPO_4. The ^{14}C-labeled D-Ins(3)P_1 and/or the ^{14}C-labeled Ins was detected in column effluent using a Flo-One\Beta Radio-Chromotography Detector (Packard Instrument Company, Downers Grove, IL, USA). To assay inhibition of IMPase by lithium chloride, the standard reaction mixture was adjusted to contain 3.0 mM LiCl.

The assays to determine the quantitative kinetic properties of IMPase were according to Webb (1992). Briefly, the assays were carried out in a coupled assay system with reaction mixtures of 1 ml containing varying concentrations of D/L-Ins(3)P_1 (0.002–400 µM), 3 mM $MgCl_2$, 100 µM 2-amino-6-mercapto-7-methylpurine riboside (MESG), 0.1 unit of purine nucleotide phosphorylase and 10 nM IMPase. The assay was monitored by measuring the time-dependent formation of the chromophoric product, 2-methyl-6-mercapto-7-methylpurine, using a Beckman DU-650 spectrophotometer set at 360 nm. The assays to quantitate the inhibition effects of different lithium concentrations were carried out in the same coupled assay system as described above with the inclusion of varying amounts of LiCl (0–4 mM).

Genomic DNA extraction, sequencing and Southern blot analysis

Genomic DNA was extracted from barley leaves using the CTAB method (Dellaporta et al. 1983). Genomic DNA was used for Southern blot analysis, and used as templates to amplify partial *IMP* genomic sequence. Primers for PCR-amplification of partial *IMP* genomic sequence obtained from leaf DNA are listed on Table 1. These primers were designed based on the *IMP* cDNA sequence. The PCR reactions contained 0.4 µM of each primer, 0.4 mM dNTPs, 500 ng of genomic DNA, and 2.5 U of JumpstartREDTaq DNA polymerase (Sigma). The PCR program was as follows; 35 cycles of denaturing at 94°C for 3:30 min, annealing at 57°C for 47 s, and extension at 72°C for 2 min. The PCR products were gel-purified using a Qiagen gel purification kit, and ligated to a pCR4-TOPO vector and transformed into *E. coli* using a pCR4-TOPO cloning kit (Invitrogen). The identity of the genomic fragment was verified by sequencing. The alignment between the genomic sequence and cDNA sequence was conducted using the Spidey program (www.ncbi.nlm.nih.gov).

For Southern blot analysis, an 800 bp partial barley *IMP* genomic sequence at the 3′-region of the *IMP* genomic gene was labeled with (α-^{32}P)dCTP using the Megapriming DNA Labeling System (Amersham company, UK). Genomic DNA was digested with *Bam*H1, *Dra*I, *Eco*RV, *Nco*I, *Sac*I and *Spe*I, respectively, and fractionated in a 0.8% agarose. The Southern blot and hybridization were conducted according to Sambrook et al. (1989).

BAC library screening, full-length *IMP* genomic sequence and promoter sequence isolation

Barley genomic library (BAC) filters were purchased from Clemson University Genomics Institute (CUGI, www.genome.clemson.edu), and hybridized using the same probe as used for the Southern blot analysis. The hybridization-positive BAC clones were purchased from CUGI. The BAC clone DNA was extracted, and PCR was conducted to confirm their identities. Three PCR products were amplified, cloned into pCR4-TOPO (Invitrogen) and sequenced. The sequences of these three PCR products were identical to those amplified from leaf DNA template. One PCR-positive BAC clone was sequenced for the IMP promoter and coding region. The initial BAC sequencing primers were designed based on the PCR products, and primer walking was then performed (Sequetech, Inc.).

Real-time RT-PCR

For the quantitative RT-PCR, primers were designed using Primer Express Software V2.0 (Applied Biosystems, Foster City, CA). The first strand cDNA was synthesized by transcriptase III (Invitrogen) in a 20 µl reaction containing 2 µg total RNA and 2.5 µM dNTPs according to the transcriptase III User Manual. Two out of the 20 µl of the first strand reaction was used as templates to amplify the second strand cDNA in a 50 µl reaction containing 25 µl of 2X SYBR Green I PCR Mix (Qiagen) and 2 µM primers in 7000 Sequence Detection Systems (Applied Biosystems). Passive dye Rox in the SYBR Green PCR Mix serves as an internal reference to adjust pipeting errors. A partial *IMP* cDNA was amplified using a forward primer 5′-GTGGATTTGGTGACGGAGACGGACAAGGC and a reverse primer 5′-AGAGAAATAAGTTATTCAGC TTGCATCTC. A partial MIPS cDNA was amplified using a forward primer 5′-ATCTCAAAGAGCAACGTGGTG GATGACATG and a reverse primer 5′-TCACTTGTA CTCCAGGATCATGTTGTTCTC. A partial α-tubulin cDNA was amplified using a forward primer 5′-AGA GAT CGATCGAGAGGCAACACTCTCCGT and a reverse primer 5′-TCACTTGTACTCCAGGATCATGTTGTTCTC, and used as an internal control for the quantification of *IMP* and MIPS mRNA levels in the real-time PCR. The cycling program was conducted with 40 cycles of denaturing at 95°C for 15 min, annealing at 57°C for 30 s, and extension at 72°C for 1 min. A validation test was performed: $\beta = 0.03$ ($\beta < 0.1$ passes the test). The relative mRNA levels were normalized to α-tubulin mRNA level, and calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). RT-PCR products were fractionated in agarose gels to confirm their identities. The single bands of expected size were purified and sequenced.

Results

Isolation and characterization of *IMP*-1 cDNA and protein

Blast searches against the barley EST database in GenBank (www.ncbi.nlm.nih.gov) using tomato (*Lycopersicon esculentum* L.) *IMP* cDNA (U39059, U39443 and U39444) as templates identified several putative barley *IMP* ESTs, including BM817370, BU966874 and AJ436586. Primers designed based on these ESTs and primers located in the library vector were used to amplify putative *IMP* cDNAs from the library. A cDNA of 1,056 bp was amplified, and sequenced. It consists of a 5′-UTR, the coding region and the complete 3′-UTR and polyA region. This barley *IMP*-1 sequence codes for a protein of 267 amino acids which is 70–75% identical and 80–85% similar to tomato *IMP*s (Fig. 1; Gillaspy et al. 1995; Styer et al. 2004).

The coding region of this putative *IMP* cDNA was subcloned into pET28b(+) and the expressed recombinant protein used for assays of catalytic properties. SDS PAGE (Fig. 2) demonstrated that induction produced a single band matching the expected size. Fractionation of the Ni-NTA eluate confirmed the presence of purified *IMP* (Fig. 2, Lane 3); a single band was found at approximately 37 kDa that appeared to be in excess as compared to the empty vector (no *IMP* gene insert plasmid control; Fig. 2, Lane 2). Also, the 37 kDa band was not found in *E. coli* BL21(DE3) cells that contained the *IMP*-inserted plasmid, but were not induced with IPTG (data not shown), an indication that the expression of *IMP* only occurred upon induction by IPTG. The presence of a 6× His tag at the C-terminus of the open reading frame of the cloned barley *IMP* allowed for further confirmation of the purified IMPase by Western blotting (Fig. 2, Lane 4). The Anti-His(C-Term) antibody detected a single band at 37 kDa corresponding to the purified IMPase and confirmed that this band contained the C-terminus 6× His tag.

Two assay systems were used to study the enzymatic properties of the barley recombinant protein (Figs. 3, 4, and Table 2). The first system assays directly for the conversion of ^{14}C-labelled Ins(3)P_1 to ^{14}C-labelled Ins, via fractionation and detection of these compounds using anion-exchange HPLC coupled to an in-line scintillation counter detector. This method allows for quick and accurate separation and detection with minimal handling, as compared with previously reported methods (Janczarek and Skorupska 2001; Gillaspy and Gruissem 2001; Ragan et al. 1988). Using this method, the barley recombinant enzyme was demonstrated to have IMPase activity, in that it catalyzed the removal of phosphate from Ins(3)P_1 to yield Ins (Fig. 3a). At a concentration of 3 mM Li^+, the activity of barley IMPase was inhibited by approximately 50% (Fig. 3b).

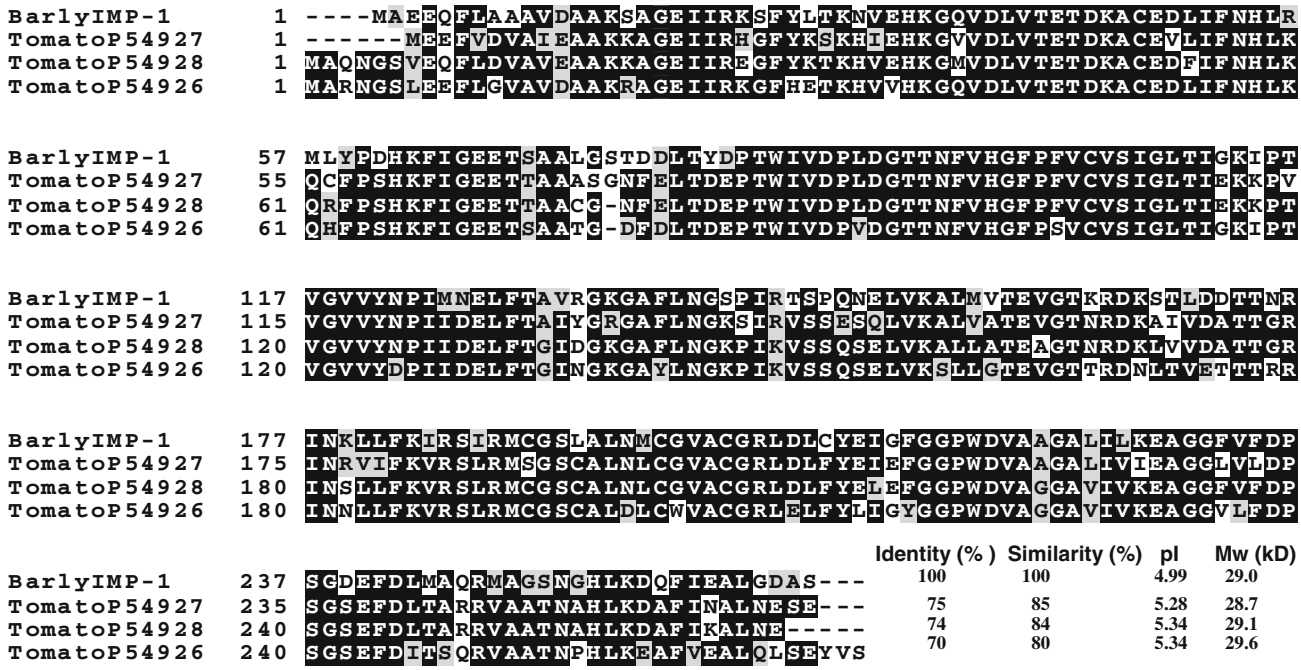


Fig. 1 Amino acid alignment of the IMPase encoded by barley *IMP*-1 as compared with the IMPases encoded by three tomato *IMP*s. The amino acids were aligned according to Worley et al. (1998; http://searchlauncher.bcm.tmc.edu/). The identities and similarities were calculated according to Tatusova and Madden (1999; www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html). The pI and Mw were calculated according to Gasteiger et al. (2005; http://us.expasy.org/tools). The three tomato *IMP* accession numbers are P5426, P5427 and P5428 (Gillaspy et al. 1995). The barley *IMP*-1 sequence accession number is AY460570

The barley recombinant IMPase was then further characterized using a coupled enzyme assay for the release of inorganic P resulting from IMPase activity; the released inorganic P serves as one substrate for purine nucleotide phosphorylase, which catalyzes the conversion of the chromogenic substrate MESG to 2-methyl-6-mercapto-7-methylpurine (Webb 1992). Using this assay the barley recombinant enzyme was found to have a K_m for D/L-Ins(3)P_1 as substrate (9.7 μM) that was 4- to 10-fold lower than the previously reported values for recombinant human and bovine IMPases or the *E. coli* SuhB enzyme (43–75 μM), and 24-fold lower than the *Rhizobium* enzyme (230 μM; Table 2). In comparison with these enzymes barley IMPase has a substantially greater affinity for this substrate, similar to that observed for the *Synechocystis* enzyme (3.4 μM). However, for those cases where published data allows for the calculation of k_{cat}, the reported k_{cat} values for the human and bovine recombinant enzymes were 19- to 20-fold greater than the observed value for the barley recombinant enzyme (Table 2). As a result the k_{cat}/K_m ratios for the human and bovine enzymes (0.56 and 0.26, respectively), are two- to fourfold greater than the ratio observed for the barley enzyme (0.13), indicating that its overall catalytic efficiency is reduced as compared with the human and bovine enzymes. Finally, the coupled enzyme assay was used to further investigate Li^+ inhibition. Li^+ inhibition increased with Li^+ concentration (Fig. 4) and the calculated K_i for Li^+ was 3.84 mM, a value approximately 10-fold greater than that observed for the human and bovine recombinant enzymes (0.3 and 0.26 mM, respectively) but similar to one reported for a *Rhizobium leguminosarum* IMP, 4.35 mM (Janczarek and Skorupska 2001).

Characterization of IMP genomic structure

Using leaf DNA as template, and primers based on the cDNA sequence, a structure of the barley genome's *IMP*-1 was derived (Fig. 5a), and compared with that of the rice and *Arabidopsis* genomes' single *IMP*s, and tomato's *LeIMP*-1. Southern blot analysis using an 807 bp segment as the probe demonstrated that the barley genome contains one copy of the *IMP* gene (Fig. 5b). Both longer film exposure to the filter and low stringency wash of the filter showed one additional weak band in the *Eco*RV and *Spe*I digestion lanes (data not shown), indicating that the barley genome contains at least one additional sequence with limited homology to the canonical *IMP* sequence, possibly representing an *IMP*-like gene.

To obtain promoter sequence and to bridge to gaps in the genomic sequence, within introns 3 and 6, an *IMP*-1-containing BAC clone (0190A09) was isolated and used for sequencing. The starting sequencing primers were designed based on the partial *IMP*-1 genomic sequences amplified

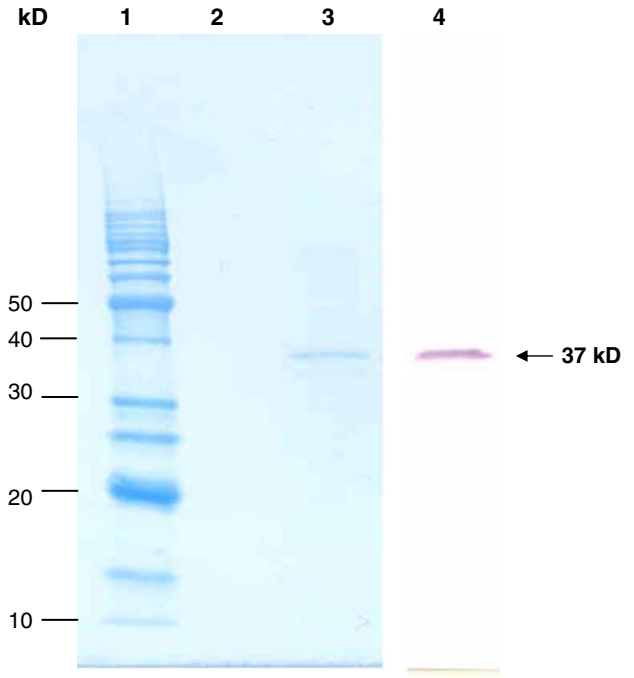


Fig. 2 SDS PAGE and Western blot analysis of the expressed barley recombinant IMPase. *E. coli* cells harboring barley *IMP*-1-containing plasmids or an empty vector pET28b(+) were cultured, induced, homogenized, and the supernatant fractionated on an Ni-NTA column. The column eluate was then fractionated in with PAGE. Lane 1, protein molecular weight markers (kDa). Lane 2, protein obtained from induction of the empty vector. Lane 3, recombinant IMPase protein obtained following induction of the barley *IMP*-1-containing vector. Lane 4, Western blot of purified barley IMP protein, probed with an anti-His-tag antibody

from the BAC clone (Fig. 6a). Primer walking was then performed. The 1.4 kb promoter and the transcribed region were sequenced (Fig. 6b).

Promoter sequence analysis (PLACE database tool; Higo et al. 1999; www.dna.affrc.go.jp/PLACE/) showed that there are two TATA sequences and one Initiator (Int)-like sequence at −663, −989 and −181, respectively. Int is a short pyrimidine-rich sequence, serves a function similar to a TATA box, and controls the transcription initiation in a TATA-less promoter in some species (Nakamura et al. 2002). Typically, TATA boxes contributing to transcription initiation are located at an average of −25 to −30, and the Int is overlapped over the initiation site at +1 (Nakamura et al. 2002). Thus it is possible that the barley *IMP*-1 promoter is TATA-less and/or Int-less.

We mapped the *IMP*-1 transcription initiation sites and determined the complete 5′-UTRs using mRNA isolated from leaves, developing seeds and roots, respectively. The mapping results showed that *IMP*-1 transcription always initiates at the same site, −181 from translation start code. Thus the *IMP*-1 promoter appears to accurately control transcription at one single site. Several putative cis-acting elements were identified in the promoter region and the 5′-UTR of the barley *IMP*-1 gene. These include elements found in genes regulated by heat, auxin, salicylic acid, cold and sugar. Thus the *IMP*-1 promoter sequence would appear to provide target sequences for the regulation of transcription. Significantly, six sugar-regulating elements including five SUREs (sugar responsive, Grierson et al. 1994) and one CGACG element (Giuliano et al. 1988; Hwang et al. 1998) are found in the promoter.

An alignment analysis of cDNA and the genomic DNA sequence showed that the barley *IMP*-1 genomic coding region consists of 10 exons and nine introns. All splice sites are in accordance to the GT (5′-end) and AG (3′-end) consensus rule (Fig. 6c). The sequence of intron 3 has not been completed, but analyses conducted to date (GenBank DQ145527 and DQ145528) indicate that this intron contains at least 13,558 bp. Due to the large size of introns 3 and 6, the barley *IMP*-1 gene is much larger than those found in other plant species. Alignment of the rice genome sequence database's single *IMP* genomic gene (AC133003) and cDNA sequence (AK071149) showed 10 exons, similar to that of the barley gene, but the longest intron (No. 6) has 1,004 nts. Of the two tomato IMP-encoding genes for which there is genomic sequence (Styer et al. 2004), *LeImp*-2 contains a single uninterrupted ORF of 798 bp, whereas *LeImp*-1 contains both exons and introns (12 and 11, respectively) spanning a total of 3,646 bp. *Arabidopsis* genome contains one single *IMP* gene (At3g02870) which has a structure similar to *LeIMP*-1.

Expression of MIPS and *IMP*-1 genes

In order to investigate spatial expression patterns of MIPS and *IMP*-1 genes, we extracted RNA from roots, developing seeds and leaves of a non-mutant barley cultivar (cv. Harrington) and conducted quantitative real-time RT-PCR using α-tubulin as an internal control. The mRNA levels of both MIPS and *IMP*-1 are higher in leaves than in developing seeds and in roots (Fig. 7a), suggesting that the de novo biosynthesis of Ins is more active in leaves than in developing seeds and in roots. A reasonable explanation is that glucose-6-P, a photosynthesis product and the only known substrate of MIPS, is most abundant in the photosynthetic source tissues (leaves). Ins produced by MIPS and *IMP*-1 activity is then available for translocation to sink tissues of a plant.

The synthesis of Ins P_6 represents a sink for Ins during seed development. Plants homozygous for barley *low phytic acid* 1-1 (*lpa*1-1), *lpa*2-1, *lpa*3-1 or M955 produce mature seed with reductions in phytic acid, as compared with non-mutant wild-type, of ∼45%, ∼30%, ∼56% and ∼94%, respectively (Dorsch et al. 2003). The block in Ins P_6 synthesis or accumulation conditioned by these

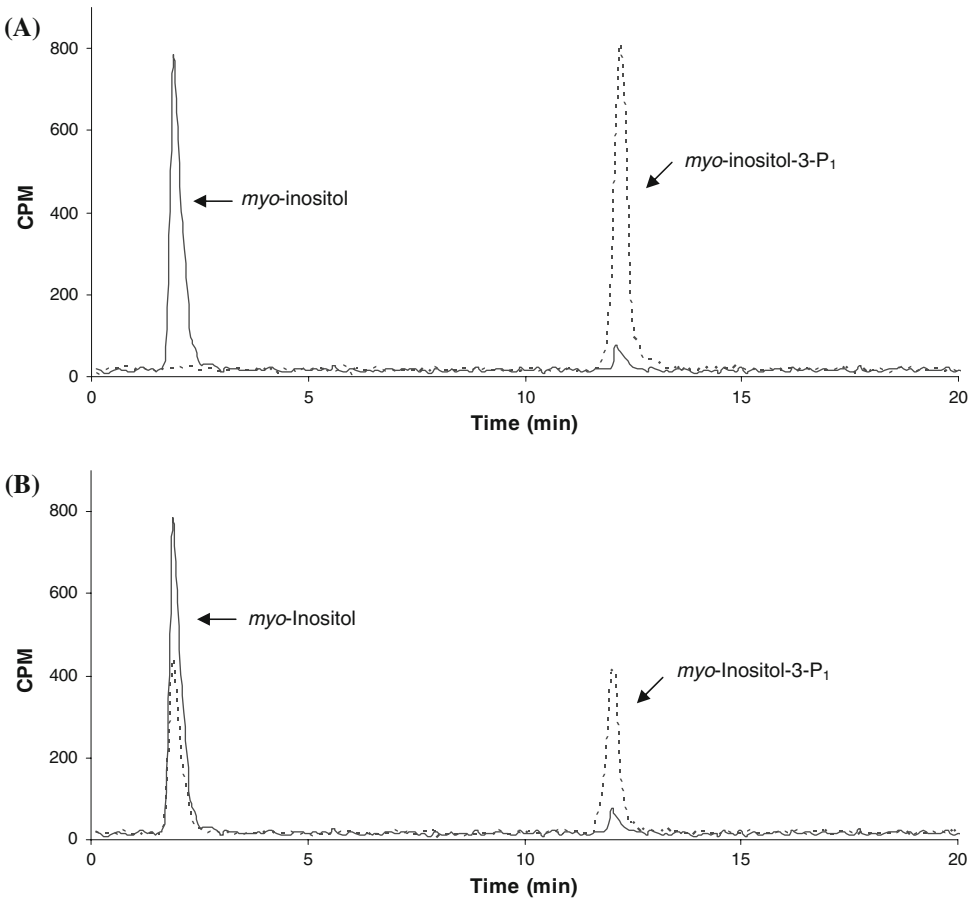


Fig. 3 Barley recombinant IMPase activity and Li+ inhibition. (**a**) Barley recombinant IMPase catalyzes the conversion of D/L- *myo*-inositol-3-P_1 to Ins. The dashed line is a fractionation of a reaction mixture (50 mM HEPES pH 7.5, 0.5 mM D/L-*myo*-inositol-3-P_1, 25 nCi ^{14}C-labeled D- *myo*-inositol(3)P_1, 3 mM $MgCl_2$) at time zero prior to addition of barley recombinant IMP. The solid line is a fractionation following incubation (37°C for 10 min in a 300-µl total volume) of the reaction mixture with 1.0 µg of purified barley recombinant IMPase. (**b**) Li+ inhibition of barley recombinant IMPase. The dashed line is a fractionation of a reaction mixture and incubation identical to that used in (**a**) except for the inclusion of 3 mM LiCl. For reference, the solid line is the same reaction products as in (**a**)

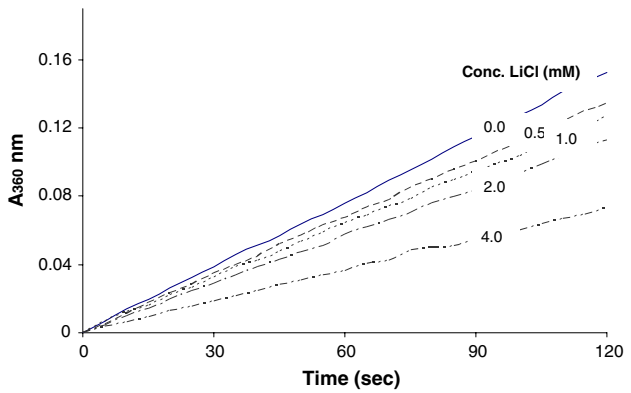


Fig. 4 Dependence of Li^+ inhibition of barley recombinant IMPase activity on concentration. Coupled-enzyme assays, as were used for Table 2, used 1.0 ml reaction volumes containing 0.2 mM D/L- *myo*-inositol(3)P_1, 3 mM $MgCl_2$, 0.1 mM 2-amino-6-mercapto-7-methylpurine riboside (MESG), 0.1 units of purine nucleotide phosphorylase, 10 nM IMPase and varying concentrations (0.0 mM, solid line; 0.5 mM, large dash line; 1.0 mM, small dash line; 2.0 mM, alternating large/small dash line; 4 mM, large/2 small dash line) of LiCl. The assay was monitored by measuring the time-dependent formation of the chromophoric product, 2-methyl-6-mercapto-7-methylpurine, using a Beckman DU-650 spectrophotometer set at 360 nm. X-axis represents incubation time

mutations is thought to be present throughout seed development, and none of these mutations are in the barley genome's single MIPS gene (Bowen et al. 2006; Larson and Raboy 1999; Roslinsky et al. 2007). As a result, Ins levels in mature whole seed homozygous for *lpa*2-1, *lpa*3-1, or M955, but not *lpa*1-1, was found to be increased two- to threefold (Karner et al. 2004).

MIPS and *IMP*-1 expression at the mRNA level was analyzed in the developing seeds (10 DAP) of plants homozygous for these mutations, using quantitative real-time PCR, and values were normalized to α-tubulin expression. MIPS expression in developing seed homozygous for *lpa*2-1, *lpa*3-1, or M955, but not *lpa*1-1, was reduced as compared to wild type, and *IMP*-1 expression in all four mutants was reduced in comparison to wild type (Fig. 7b). The level of reduction appeared fairly proportional to both the decrease in Ins P_6 and increase in Ins.

Discussion

The ancestral eukaryotic genome probably contained one copy of the *IMP* gene (Shamir et al. 2001). Studies of

Table 2 Kinetic parameters for recombinant inositol monophosphatases obtained using *myo*-inositol-3-P_1 as substrate

Organism	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})	k_{cat} (s^{-1})	Reference
Human	75 ± 3	36.1 ± 1	–	McAllister et al. (1992)
Human	43 ± 8	–	24.1 ± 1.3	Pollack et al. (1993)
Bovine	95 ± 20	40.0 ± 3.4	–	Leech et al. (1993)
Bovine	100	–	26	Strasser et al. (1995)
Synechocystis	3.4 ± 0.3	0.0333 ± 0.0003	–	Patra et al. (2007)
Rhizobium	230 ± 10	3.27 ± 0.02	–	Janczarek and Skorupska (2001)
E. coli SuhB	69 ± 10	3.38 ± 0.06	–	Chen and Roberts (2000)
Barley	9.7 ± 1.1	0.0903 ± 0.002	1.29 ± 0.1	Present study

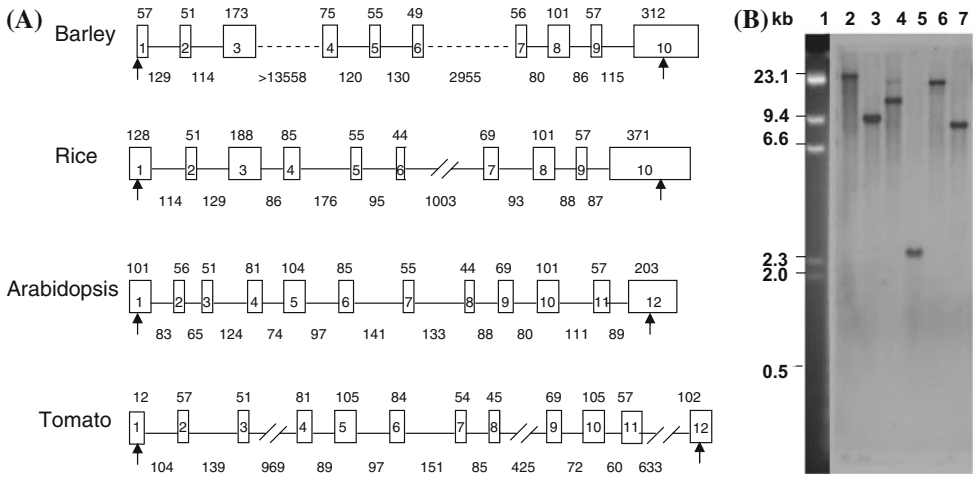
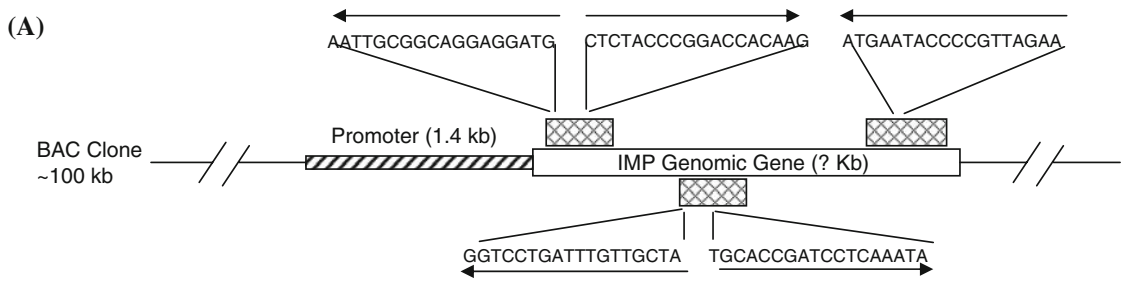


Fig. 5 Barley *IMP*-1 genomic structure and Southern blot determination of gene copy number. (**a**) Barley *IMP*-1 exon–intron structure was based on the alignment between mRNA sequence AY460570 and *IMP*-1 partial genomic DNA sequence amplified from barley leaf tissues. Boxes represent exons and lines represent introns. ↑ represents the first methionin codon or the translation stop codon. The dashed line for barley introns 3 and 6 reflect that sequencing is incomplete. For more information about intron # 3 and 6 (see Fig. 6c). Rice *IMP* exon–intron structure was based on the alignment between mRNA sequence AK071149 and genomic sequence AC133003 nucleotide No. 182773–185792. *Arabidopsis IMP* exon–intron structure was based on the alignment between mRNA sequence AY035150 and genomic sequence AC018363 nucleotide No. 56541–58699. Tomato *IMP* exon–intron structure was based on the alignment between mRNA sequence U39444 and *LeIMP*-1 genomic DNA sequence AY227666 (Styer et al. 2004). (**b**) Southern blot analysis indicates the barley genome contains one copy of the *IMP* gene. (Lane 1) Molecular size markers (Lambda/*Hin*dIII fragments). (Lanes 2–7) Barley genomic DNA was extracted from leaves, digested with the following restriction enzymes and fractionated in an agarose gel; (Lane 2) *Bam*H1, (Lane 3) *Dra*I, (Lane 4) *Eco*RV, (Lane 5) *Nco*I, (Lane 6) *Sac*I and (Lane 7) *Spe*I. The 800 bp partial barley *IMP*-1 genomic sequence at the 3′-region of the *IMP*-1 gene was labeled with (α-^{32}P)dCTP as a probe for the filter hybridization

mammalian genomes to date indicate that they contain two *IMP* genes, *IMPA1* and *IMPA2*, that resulted from a duplication event that occurred after the evolutionary separation of the vertebrate lineage (Shamir et al. 2001). The two mammalian *IMP* genes display different patterns of expression and encode proteins with different physical, catalytic and regulatory properties (Arai et al. 2007; Ohnishi et al. 2007). Thus, in this case, the duplication of the ancestral eukaryotic gene provided substrate for the evolution of new functionality, a case of "neo-functionalization" (Lynch and Katju 2004). Plant genomes studied to date contain from one (barley and *Arabidopsis*) to three (tomato) *IMP* copies (Gillaspy et al. 1995; Torabinejad and Gillaspy 2006). The gene duplication event that gave rise to the tomato's intronless *LeIMP*-2 copy occurred after the divergence of the lineages that gave rise to *Arabidopsis* and tomato (Styer et al. 2004). Therefore there is no correlation between copy number and genome size, since *Arabidopsis* has a very small genome (157 MB) whereas barley has a very large genome (~5,000 MB), nor is there a clear relation between copy number and fitness, since barley displays a wide range of adaptation, nor is there strong evidence for the evolution of new functionality.

In the case of higher plants, new functionality may have been provided by the inheritance of both *IMP* and *IMP*-like genes. The IMP genes encode proteins that have been documented to possess both IMPase and L-Gal-1-P phosphatase activities (Parthasarathy et al. 1997; Conklin et al.



(B)

```
----------AGCTTGTTAGTGCATGAAAGCACAAGCAGACAAGAAGCACACCTCCCGCCAATTCGAGGTGGG
                                                    Heat Shock RE

-1232 CGATCAAGTTTTCCTCAAACTTTAACCCTACATTCAGTCGTCCGTCGCTCCGCGAGTGAACCACAAGCTCTCGTTCAAGTTCTTTGG
                  Auxin RE

      GCCGTTCCCCATCATCGCCAAGGTTAACGATGTCACGTACAAGTTGTAGTTGCTCGGTTGTCCATTCGATCTTTCACGTCCCTGAGTT
-1056 GAAGAGGGCACTACTGCCGCGTACAACCGTTTCTTCGCACTTGCCAATTCCTGACGATGCACTTGCTATATCAGTGGAGATCCTTCA
                                                                  -989
      ATCGCGTTAGCGCAAGACAGCCGGGGCCATGGTCGAACATGTCAAGGTCCGTTGATCCGACAACTCTGTTGTGAAAAATACATGGA
                                                    Gibberellin RE

-880  AAGATAAAAACTGTTTTACAAGCTCGCTTTCCGACAACCAAGACTTGGGGCAAACCTATGCACAAGGGAAGGGGCATGTGAGCGC
                  Low Temperature RE

      GGCTGACATACGTGACCCACCTGTTTGCACTACCGGGCCAAGCGTGGGGAGGCCGAGGCACAACCCGCGGCCCAACTTGCATGTGA
-704  CCGGTCTCGAGTGGACCTCGTGAAGCCCAAGGCGCGTCGGTATATAAGCGCCTGCCACCGAGGGACTGGATATCTAGAGGGTGCTT
                                    -663
      GGATACGTTTAAGTCCCATGACTAAAAATAGTGAGACTAAAACTTGTTAGCCTTACCCATGCTTGGATCCAAATACTAAAGAGACT
                  Sugar RE

-528  AAAATCAAGTTAATGAGCATTTATTATCCTGCAAACCCTCCAATCCAAAACTTGTCTGAGGAGTTAAATGAGGAGAGAGAGGACTA
                                    Heat Shock RE

      ATGCACATTTTAGTAGGGGTACCCGTGACTAAAAGATTTTAGCCTCAAGACTAGTTTTAGAGGGTGTTTGAATACGTTTTGGTCCCA
                  Sugar RE

-352  TGACTAAAAGTAGTGAGACTAAAACTTGCTAGCCTCATCCATGCTTGGATCCAAATATTAAAGAGACTAAAATCAAGTTAATGAGC
      Sugar RE

      ATTTATTATCCTGCAAACCTTTCAATCCAGAACTCGTGTGAGGAGTTAAATGAGGAGAGAGAGGACTAATGCACATTTTAGTAGGGGTA
-176  CCCTGACTAAAAGATTTTAGTCTTAAGACTAATTTTAGCCTCTCTTTAGTCAGGGGTGCTTGAAACTTTAGCATCCTAAAAAGA
      Sugar RE                                                    Auxin RE

      CTAGTTTTAGTCAGACTAGTTTTAGTCCATTGGATCTAAGCACCCTCTTAGCCTCTATTTAGTCAGGGTGAAATTTAGCCTTCTGAG
+1    AGTTTTAGTTGATTGGATCCAATCACCCTCGTAGCCCGGCGACGACGGCTACCTGTTCTTCCCCATCTTTCCATCCCCTTTCCCAATTC
                                    CGACG element

      GTGTAATCGTGACTCGAATCGTGTGCCTAATCAATAGATCGAGAGCATCCACTACTAGTCCCTAACATCTGGAGGCGGGAGATTCGGA
            Sugar RE

      AGCCATG (Methionin)
```

Fig. 6 Barley *IMP*-1-containing BAC clone sequencing strategy and sequences of the *IMP*-1 promoter and the transcribed region. (**a**) Sequencing strategy. Primers were designed based on the partial sequence amplified from the BAC clone and served as the start points for primer walking for the promoter and transcribed region sequences. (**b**) Promoter sequence. The 1.4 kb promoter region was sequenced and analyzed using PLACE data basetool (Higo et al. 1999, www.dna.affrc.go.jp/PLACE/). The promoter sequence has been submitted to the GenBank. Accession # is DQ145527. (**c**) The IMP genomic sequence. More than 15 kb of the transcribed region was sequenced and analyzed using Spidey program (www.ncbi.nlm.nih.gov). It consists of 10 exons and 9 introns. The intron # 3 and 6 have >13 kb and about 3 kb nts, respectively. The sequence has been submitted to the GenBank. Accession numbers are DQ15527 and DQ145528

2006; Laing et al. 2004), and may be bifunctional in vivo, impacting and perhaps contributing to the coordination of Ins and galactose metabolism, the latter important to ascorbic acid synthesis in higher plants. However, this bifunctionality predates the divergence of mammals and plants, since it is observed in both lineages.

(C)

ATGGCGGAGGAGCAGTTCCTGGCCGTAGCGGTGGACGCCGCCAAGAGCGCCGGCGAGgtaatcctcctaatgaactcgatccgtccctccatcctcctgccgca

M A E E Q F L A A A V D A A K S A G E **(Intron # 1)**

attgtttctcaggcccactgctcctgatggattcgcgattgcctgttcactcattctgtgaccgatttgtacgccatgcagATCATCCGCAAGAGCTTTTACCTAACCAAGAATGTGGA

I I R K S F Y L T K N V E

GCACAAGGGCCAGgtctctcctctcgcctcttcttttccccacgcatgttacctccttttgacgccgctgtccttgattggattgcaatctaatcgtcacggtgctatggattgtggctgctgcagGTGGA

H K G Q **(Intron # 2)** V D

TTTGGTGACGGAGACGGACAAGGCCTGCGAGGATCTCATCTTCAACCACCTCCGGATGCTCTACCCGGACCACAAGTTCATCGGCGAGGA

L V T E T D K A C E D L I F N H L R M L Y P D H K F I G E E

GACGTCTGCAGCCCTTGGCTCCACCGATGACCTCACCTATGACCCTACCTGGATAGTCGACCCCCTCGATGGCACCACgtaacgacgatgttcta

T S A A L G S T D D L T Y D P T W I V D P L D G T T

gtatgcgcactaccattgttttcttgctttcttctaagaac/**Intron#3>13kb/**gccatctttcaaaaaggcaaagtccctttcttcatcatgcaattttctttttcttattctaaaattgtgcacctttttttaaatatttcag

CAATTTCGTTCATGGCTTTCCTTTTGTGTGCGTCTCGATTGGCCTCACCATTGGGAAGATTCCCACTGTTGGAGTTGTGTACAACCCCATC

N F V H G F P F V C V S I G L T I G K I P T V G V V Y N P I

ATGAATGAGgtaaacattgttactctatttaatttttcttgttttatttgaggatcggtgcaatataatctgggcaaaggaacctggaaaggttttgagtccgatttcatgttttttcttcaattagCTTTTCAC

M N E **(Intron # 4)** L F T

AGCTGTTCGTGGAAAAGGTGCTTTCCTTAATGGTTCTCCAATTAGAAgtaagcacatttttgttgattaggtcctgatttgttgctatacaacagaagaagcaagtcaagatt

A V R G K G A F L N G S P I R T **(Intron # 5)**

acacagttctttttcggattgctcgcatgtgttaatttcaatatacacctctcatgtgtagCATCGCCTCAAAATGAGTTGGTGAAGGCTCTTATGGTGACAGAGGTAGG

S P Q N E L V K A L M V T E V G

gttacagacactgtcaaagattatcatttccatgtcattcgc/ **Intron # 6:~3kb/**aatctaccggaatgttcaattaattaccatattaattgagcaggtag

GACCAAAAGAGACAAGTCCACTTTGGATGATACAACCAACAGAATTAATAAGTTACTATTCAAGgtaaatcctgtaaagtaacatttttatcagagttatttg

T K R D K S T L D D T T N R I N K L L F K **(Intron # 7)**

tagcatgtcaatattctaacggggtattcatttttttcagATTAGATCTATACGTATGTGTGGCTCTTTGGCTCTAAACATGTGTGGAGTTGCTTGTGGTAGGCTA

I R S I R M C G S L A L N M C G V A C G R L

GATTTGTGTTATGAGATCGGTTTTGGTGGCCCCTGgtattgttaccaaattattaacgattgtcttacatggaaaatatataaaccaaatgtaacagctaattgttatttttcctctagG

D L C Y E I G F G G P W **(Intron # 8)**

GATGTGGCTGCTGGAGCTTTGATTCTAAAGGAAGCAGGGGGTTTTGTTTTTGATCCgtaagttgtcaaatttgtcaatactgtatggctggctcttgctatcatagtac

D V A A G A L I L K E A G G F V F D P **(Intron # 9)**

ataatttgatgagatttttttccatttcttgttgtacattttctttcattttgacttttagGAGCGGTGATGAGTTTGATCTGATGGCGCAAAGAATGGCCGGATCAAATGGCCAC

S G D E F D L M A Q R M A G S N G H

CTCAAGGATCAGTTCATCGAAGCATTGGGAGATGCAAGCTGAATAACTTATTTCGTCAAGTAGAATGAAAGAATGTACGATGGCCCCACCA

L K D Q F I E A L G D A S *

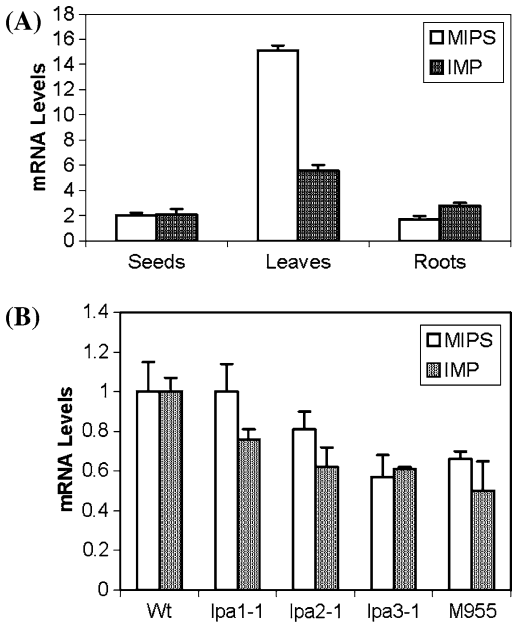
ATAAGTAATTAAGGGCTAATTTTCGTGTAGTTCTACGTGAACGCTATGCATATTTTGCAAACATGGTGGATGTAATGACATGTCGATATATT

GCTCGTTTTATTGACCATGCAAGTAATTCATGCTTGCAGGCGATGAAATGGCTCGTAAGCTACAATTCCCTCCT

Fig. 6 continued

In higher plants, the new functionality provided by the expression of both *IMP* and *IMP*-like genes, may rather represent a form of "sub-functionalization", where the relative contribution to IMPase and L-Gal-1-P phosphatase activities in various tissues and developmental stages is subdivided between the various genes. For example, it may be that the *IMP* gene products in vegetative tissues function more as L-Gal-1-P phosphatases, whereas in seed tissues they contribute to Ins metabolism. The *IMP*-like genes perhaps may be more important to constitutive Ins synthesis, perhaps as part of the "coupled" MIPS/IMPase pathway, or may act on D-Ins(1)P_1, thus functioning in Ins(1,4,5)P_3 signal recycling.

Studies of the kinetic parameters of plant IMPases are limited in number. Gillaspy et al. (1995) reported that the three tomato IMPases are Mg^{2+} dependent and inhibited by Li^{+}, but studies of the tomato enzyme's kinetic parameters such as K_m and V_{max} have not been reported. More recently Islas-Flores and Villanueva (2007) purified an IMPase from soybean embryos and reported that this enzyme, like



Level Relative to Wt					
Inositol Phosphate	1.00	0.55	0.70	0.44	0.06
Inositol	1.00	1.00	2.54	2.02	3.05

Fig. 7 Expression of *IMP*-1 in barley tissues. (**a**) Spatial (leaf, seed and root) expression analysis of MIPS and *IMP*-1 genes. Leaf and root RNA was extracted from 4 weeks old plants. Seed RNA was extracted from seed tissues at an early development stage (10 DAP). The relative mRNA levels were calculated using quantitative RT-PCR and $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). (**b**) Expression analyses of MIPS and *IMP*-1 genes in wild type and *low phytic acid* (*lpa*) barley genotypes. RNA was extracted from seed tissues of early development stage of wild type, *lpa*1-1, *lpa*2-1, *lpa*3-1, and M 955 isolines. Quantitative real-time PCR was conducted as described in the section "Materials and methods." Levels of inositol and inositol phosphates, including all inositol phosphates detected with HPLC, in mature whole seed of each genotype, were from Dorsch et al. (2003) and Karner et al. (2004). Each bar represents the mean of three replicates with the indicated standard deviation of the mean

many IMPases, has a broad substrate specificity including activity against Ins P_6, concluding that the soybean IMPase may play an important role in phosphate metabolism during germination. Islas-Flores and Villanueva (2007) reported a K_m for the soybean IMPase against sodium pyrophosphate, so the value obtained (235 μM) cannot be directly compared with IMPase K_m values against Ins(3)P_1, as reported here and in most other studies.

Recently Patra et al. (2007) isolated sequences encoding an IMPase from the cyanobacterium *Synechocystis* sp. PCC6803, and studied the catalytic properties of the recombinant protein. This enzyme is similar to other IMPases in that its activity is Mg^{2+} dependent and inhibited by Li^+, and in that it recognizes a broad range of substrates including D/L-Ins(3)P_1, fructose1,6-bisP, fructose 6-P, fructose 2,6-bisP, and sucrose 6-P, but its activity against these later substrates was less than 5% of the maximum activity found with D/L-Ins(3)P_1. The most notable finding of Patra et al. (2007) was that the *Synechocystis* recombinant IMPase displayed a relatively high affinity for D/L-Ins(3)P_1 (K_m = 3.4 μM), coupled with a relatively low maximal velocity (V_{max} = 0.0333 μmol min^{-1} mg^{-1}), as compared with the K_m and V_{max} values observed for recombinant IMPases from mammalian sources (Table 2). Patra et al. (2007) conclude that these properties make the *Synechocystis* enzyme ideal to serve in a coupled reaction with MIPS for the production of Ins.

Data reported here indicate that the kinetic properties of the barley IMPase are similar to those of the *Synechocystis* enzyme, in that relative to data reported for the mammalian enzymes, it has a higher affinity for D/L-Ins(3)P_1 but a lower overall reaction rate. This is supported by comparisons where available of k_{cat} values, where $k_{cat} = V_{max}$/total enzyme concentration. The barley IMPase k_{cat} is approximately 20-fold less than values obtained for human or bovine IMPases (Table 2). The barley *IMP-1*-encoded IMPase's affinity for Ins(3)P_1 as substrate is greater than the reported affinities of the *Arabidopsis* "L-Gal-1-P phosphatases/IMPases" for L-Gal-1-P (Laing et al. 2004). Thus its catalytic properties indicate that the barley enzyme could function in Ins synthesis, but its activity against L-Gal-1-P and its role in ascorbic acid synthesis requires further study. In any case, relative differences in catalytic properties, even "fold-differences" in K_m or V_{max} values, do not definitively point to different biological roles. More definitive results would come from genetic analyses. Phenotypic characterization, including measurement of tissue Ins and ascorbic acid levels in lines homozygous for recessive alleles of these various genes and gene combinations, would greatly illuminate the relative roles of these genes and gene products. This type of genetics approach definitively demonstrated that *Arabidopsis VTC4* encodes a protein with L-Gal-1-P phosphatase activity important to vegetative ascorbic acid synthesis, but the role of this protein as an IMPase in vegetative or seed Ins metabolism has not been reported. Similarly, while barley IMP-1 is expressed in both vegetative and seed tissues, what contribution does this make to net seed Ins synthesis and seed Ins P_6?

The proteins encoded by *IMP* genes are relatively highly conserved among eukaryotes, with sequence similarity typically 70% or greater in species ranging from human, mouse, *Xenopus*, and *Drosophila*, to tomato, *Arabidopsis* and barley (Shamir et al. 2001; Fig. 1). Divergence in genomic sequence is therefore primarily in promoter sequences, in intron sequence and size, and to a lesser extent in intron/exon structure, as is evident for the plant species in Fig. 5a. The most notable difference in the genes in Fig. 5a is the presence of two large introns in the barley gene, as compared either with the relatively closely related rice, or more distantly related tomato and *Arabidopsis*

genes. The origin of the barley *IMP*-1 gene's large Intron 3, which appeared after the relatively recent divergence of rice and barley, is unknown at present. There is a precedent for the presence of large introns in closely related members of an *IMP* gene family. The two copies of *IMP* genes in the human genome are nearly identical, except that the human *IMPA2* gene contains a 10 kb intron 3 whereas the *IMPA1* copy contains a 1.4 kb intron 3, and the *IMPA1* copy contains a 10.6 kb intron 7 whereas *IMPA2* contains a 0.7 kb intron 7 (Shamir et al. 2001; Sjøholt et al. 1997, 2000). The possible impact of these large introns on gene expression or organismal fitness is unknown.

Observations from sequence analysis of the barley *IMP*-1 promoter are reminiscent of observations made following sequence analysis of the human *IMPA1* and *IMPA2* genes. Both the barley gene and the human *IMPA2* genes contain promoters and/or other untranslated sequences that have characteristics of house-keeping genes as well as cis-acting response elements important to metabolic regulation (Yoshikawa et al. 2000). Thus both the barley *IMP*-1 and the human *IMPA2* genes may be "TATA-less" while also containing regulatory elements such as the barley promoter's sugar-response elements (SUREs), and the human *IMPA2* gene's "inositol/choline responsive element" (ICRE; Sjøholt et al. 2000). The presence of SUREs may indicate that barley *IMP*-1 gene expression is regulated by sugar status in plant cells, a regulation in keeping with its potential role in both Ins and galactose metabolism. Analysis of *IMP*-1 expression during seed development in barley *lpa* mutants indicates sensitivity to Ins levels. These analyses indicate the barley *IMP*-1 expression is similar to human *IMPA* in that it is constitutive, but that this constitutive expression may also be modulated in response to metabolic or stress-response needs. Further studies are needed to test the responsiveness of the barley *IMP* promoter to varying levels of sugars and Ins.

Acknowledgements The authors would like to thank Justin Wheeler and David Bowen for help with the greenhouse work, and Phillip Bregitzer and Ann Sturbaum for help with the BAC library filter hybridization. This work was supported in part by the National Science Foundation (Project No. 0110748).

References

Arai R, Ito K, Ohnishi T, Ohba H, Akasaka R, Bessho Y, Hanawa-Suetsuga K, Yoshikawa T, Shirouzu M, Yokoyama S (2007) Crystal structure of human *myo*-inositol monophosphatase 2, the product of the putative susceptibility gene for bipolar disorder, schizophrenia, and febrile seizures. Proteins 67:732–742

Berridge G, Downes CP, Hanley MR (1989) Neural and developmental actions of lithium; a unifying hypothesis. Cell 59: 411–419

Bowen DE, Guttieri MJ, Peterson K, Peterson K, Raboy V, Souza EJ (2006) Phosphorus fractions in developing seeds of four low phytate barley (*Hordeum vulgare* L.) genotypes. Crop Sci 46:2468–2473

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal Biochem 72:248–254

Chen I-W, Charalampous FC (1966) Biochemical studies on inositol IX. D-Inositol 1-phosphate as intermediate in the biosynthesis of inositol from glucose 6-phosphate and characteristics of two reactions in this biosynthesis. J Biol Chem 241:2194–2199

Chen L, Roberts MF (2000) Overexpression, purification, and analysis of complementation behavior of *E. coli* SuhB protein: comparison with bacterial and archaeal inositol monophosphatases. Biochemistry 39:4145–4153

Chirgwin JM, Przybyla AE, MacDonald RJ, Rutter WJ (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18:5294–5299

Cohen JD, Bandurski RS (1982) Chemistry and physiology of the bound auxins. Annu Rev Plant Physiol 33:403–430

Conklin PL, Gatzek S, Wheeler GL, Dowdle J, Raymond MJ, Rolinski S, Isupov M, Littlechild JA, Smirnoff N (2006) *Arabidopsis thaliana* VTC4 encodes L23 galactose-1-P phosphatase, a plant ascorbic acid biosynthetic enzyme. J Biol Chem 281:15662–15670

Dellaporta Sl, Wood J, Hicks JB (1983) A plant DNA minipreparation: version II. Plant Mol Biol Rep 1:19–21

Diehl ER, Whiting P, Potter J, Gee N, Ragan CI, Linemeyer D, Schoepfer R, Bennett C, Dixon AFR (1990) Cloning and expression of bovine brain inositol monophosphatase. J Biol Chem 265:5946–5949

Dorsch AJ, Cook A, Young AK, Anderson NJ, Bauman TA, Volkmann JC, Murthy PNP, Raboy V (2003) Seed phosphorus and inositol phosphate phenotype of barley low phytic acid genotypes. Phytochemistry 62:691–706

Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Appel RD, Bairoch A (2005). Protein identification and analysis tools on the ExPASy server. In: Walker JM (ed) The proteomics protocols handbook. Humana Press, NJ

Gillaspy GE, Gruissem W (2001) Li^+ induces hypertropy and down regulation of *myo*-inositol monophosphatase in tomato. J Plant Growth Regul 20:78–86

Gillaspy GE, Keddie JS, Oda K, Gruissem W (1995) Plant inositol monophosphatase is a lithium-sensitive enzyme encoded by a multigene family. Plant Cell 7:2175–2185

Giuliano G, Pichersky E, Malik VS, Timko MP, Scolnic PA, Cashmore AR (1988) An evolutionarily conserved protein binding sequence upstream of a plant light-regulated gene. Proc Natl Acad Sci USA 85:7089–7093

Grierson C, Du J-S, de Torres Zabala M, Beggs K, Smith C, Holdsworth M, Bevan MW (1994) Separate cis sequences and trans factors direct metabolic and developmental regulation of a potato tuber storage protein gene. Plant J 5:815–826

Higo K, Ugawa Y, Iwamoto M, Korenaga T (1999) Plant cis-acting regulatory DNA elements (PLACE) database. Nucleic Acids Res 27:297–300

Hitz WD, Carlson TJ, Kerr PS, Sebastian SA (2002) Biochemical and molecular characterization of a mutation that confers a decreased raffinosaccharide and phytic acid phenotype on soybean seeds. Plant Physiol 128:650–660

Hwang Y-S, Karrer EE, Thomas BR, Chen L, Rodriguez RL (1998) Three cis elements required for rice-amylase Amy3D expression during sugar starvation. Plant Mol Biol 36:331–341

Ishitani M, Majumder AL, Bornhouser A, Michalowski CB, Jensen RG, Bohnert HJ (1996) Coordinate transcriptional induction of

myo-inositol metabolism during environmental stress. Plant J 9:537–548

Islas-Flores I, Villanueva MA (2007) Inositol-1 (or 4)-monophosphatase from *Glycine max* embryo axes is a phosphatase with broad substrate specificity that includes phytate dephosphorylation. Biochim Biophys Acta 1770:543–550

Janczarek M, Skorupska A (2001) The *Rhizobium leguminosarum* bv. Trifolii pssB gene product is an inositol monophosphatase that influences exopolysaccharide synthesis. Arch Microbiol 175:143–151

Karner U, Peterbauer T, Raboy V, Jones DA, Hedley CL, Richter A (2004) *myo*-Inositol and sucrose concentrations affect the accumulation of raffinose family oligosaccharides in seeds. J Exp Bot 55:1981–1987

Laing WA, Bulley S, Wright M, Cooney J, Jensen D, Barraclough D, MacRae E (2004) A highly specific L-galactose-1-phosphate phosphatase on the path to ascorbate biosynthesis. Proc Natl Acad Sci USA 101:16976–16981

Larson SR, Raboy V (1999) Linkage mapping of maize and barley *myo*-inositol 1-phosphate synthase DNA sequences: correspondence with a low phytic acid mutation. Theor Appl Genet 99:27–36

Leech AP, Baker GR, Shute JK, Cohen MA, Gani D (1993) Chemical and kinetic mechanism of inositol monophosphatase and its inhibition by Li^+. Eur J Biochem 212:693–704

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25:402–408

Loewus FA, Murthy PPN (2000) *myo*-Inositol metabolism in plants. Plant Sci 150:1–19

Lynch M, Katju V (2004) The altered evolutionary trajectories of gene duplicates. Trends Genet 20:544–549

Majee M, Maitra S, Dastidar KG, Pattnaik S, Chatterjee A, Hait NC, Das KP, Majumder AL (2004) A novel salt-tolerant L-*myo*-inositol-1-phosphate synthase from *Porteresia coarctata* (Roxb.) Tateola, a halophytic wild rice. J Biol Chem 279:28539–28552

McAllister G, Whiting P, Mammond AE, Knowles RM, Atack RJ, Bailey JG, Maigetter R, Ragan CI (1992) cDNA cloning of human and rat brain *myo*-inositol monophosphatase. Biochemistry 284:749–754

Nakamura M, Tsunoda T, Obokata J (2002) Photosynthesis nuclear genes generally lack TATA-boxes: a tobacco photosystem I gene responds to light through an initiator. Plant J 29:1–10

Nelson DE, Rammesmayer G, Bohnert HJ (1998) Regulation of cell-specific inositol metabolism and transport in plant salinity tolerance. Plant Cell 10:753–764

Ohnishi T, Ohba H, Seo K-C, Im J, Sato Y, Iwayama Y, Furuichi T, Chung S-K, Yoshikawa T (2007) Spatial expression patterns and biochemical properties distinguish a second *myo*-inositol monophosphatase, IMPA2 from IMPA1. J Biol Chem 282:637–646

Parthasarathy R, Parthasarathy L, Vadnal R (1997) Brain inositol monophosphatase identified as a galactose 1-phosphatase. Brain Res 778:99–106

Patra B, Dastidar KG, Maitra S, Bhattacharyya J, Majumder AL (2007) Functional identification of sll1383 from *Synechocystis* sp PCC 6803 as L-*myo*-inositol 1-phosphate phosphatase (EC 3.1.3.25): molecular cloning, expression and characterization. Planta 225:1547–1558

Pollack SJ, Knowles JR, Atack HB, Broughton HB, Ragan CI, Osborne SA, McAllister G (1993) Probing the role of metal ions in the mechanism of inositol monophosphatase by site-directed mutagenesis. Eur J Biochem 217:281–287

Raboy V (2007) Seed phosphorus and the development of low-phytate crops. In: Turner BL, Richardson AE, Mullaney EJ (eds) Inositol phosphates linking agriculture and the environment. CABI, Oxfordshire

Ragan CI, Watling KJ, Gee NS, Aspley S, Jackson RG, Reid GG, Baker R, Billington DC, Barnaby RJ, Leeson PD (1988) The dephosphorylation of inositol 1,4-bisphosphate to inositol in liver and brain involves two distinct Li^+-sensitive enzymes and proceeds via inositol-4-phosphate. Biochem J 249:143–148

Roslinsky V, Eckstein PE, Raboy V, Rossnagel BG, Scoles GJ (2007) Molecular marker development and linkage analysis in three low phytic acid barley (*Hordeum vulgare*) mutant lines. Mol Breed 20:323–330

Sambrook J, Fritrch EF, Maniatis T (1989) Molecular cloning. Cold Spring Harbor Laboratory Press, New York

Shamir A, Sjøholt G, Ebstein RP, Agam G, Steen VM (2001) Characterization of two genes, *Impa1* and *Impa2* encoding mouse *myo*-inositol monophosphatases. Gene 271:285–291

Shi J, Wang H, Hazebroek J, Ertl DS, Harp T (2005) The maize low-phytic acid 3 encodes a *myo*-inositol kinase that plays a role in phytic acid biosynthesis in developing seeds. Plant J 42:708–719

Sjøholt G, Molven A, Løvlie R, Wilcox A, Sikela JM, Steen VM (1997) Genomic structure and chromosomal location of a human *myo*-inositol monophosphatase gene (*IMPA*). Genomics 45: 113–122

Sjøholt G, Gulbrandsen AK, Løvlie R, Berle JØ, Molven A, Steen VM (2000) A human *myo*-inositol monophosphatase gene (*IMPA2*) localized in a putative susceptibility region for bipolar disorder on chromosome 18p11.2: genomic structure and polymorphism screening in manic-depressive patients. Mol Psych 5:172–180

Smirnoff N, Cumbes QJ (1989) Hydroxyl radical scavenging activity of compatible solutes. Phytochemistry 28:1057–1060

Strasser F, Pelton PD, Ganzhorn AJ (1995) Kinetic characterization of enzyme forms involved in metal ion activation and inhibition of *myo*-inositol monophosphatase. Biochem J 307:585–593

Styer JC, Keddie J, Spence J, Gillaspy GE (2004) Genomic organization and regulation of the *LeIMP-1* and *LeIMP-2* genes encoding *myo*-inositol monophosphatase in tomato. Gene 326:35–41

Tatusova TA, Madden TL (1999) Blast 2 sequences, a new tool for comparing protein and nucleotide sequences. FEMS Microbiol Let 174:247–250

Torabinejad J, Gillaspy GE (2006) Functional genomics of inositol metabolism. In: Majumder AL, Biswas BB (eds) Biology of inositols and phosphoinositides. Springer, New York

Wanek R, Richter A (1997) Biosynthesis and accumulation of D-ononitol in *Vigna umbellata* in response to drought stress. Physiol Plant 101:416–424

Webb MR (1992) A continuous spectrophotometric assay for inorganic phosphate and for measuring phosphate release kinetics in biological systems. Proc Natl Acad Sci USA 89:4884–4887

Worley KC, Culpepper P, Wiese BA, Smith RF (1998) BEAUTY-X: enhanced BLAST searches for DNA queries. Bioinformatics 14:890–891

Yoshida KT, Wada T, Koyama H, Mizobuchi-Fukuoka R, Naito S (1999) Temporal and spatial patterns of accumulation of the transcript of *myo*-inositol-1-phosphate synthase and phytin-containing particles during seed development in rice. Plant Physiol 119:65–72

Yoshikawa T, Padigaru M, Karkera JD, Sharma M, Berrettini WH, Esterling LE, Detera-Wadleigh SD (2000) Genomic structure and novel variants of *myo*-inositol monophosphatase 2 (*IMPA2*). Mol Psych 5:165–171